

A Mouse Model for the Molecular Characterization of Brca1-Associated Ovarian Carcinoma

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Abstract

Little is known about the mechanisms that underlie Brca1-associated ovarian tumorigenesis, mainly due to the lack of an appropriate experimental model. We developed genetically defined primary mouse ovarian surface epithelial (OSE) cell lines in which the loss of functional Brca1 and p53 recapitulates the events that are thought to occur in early ovarian cancer development in patients with Brca1 mutations. This system allows for the introduction of additional oncogenes that are thought to cooperate with the loss of Brca1 and p53 to induce tumorigenesis. We showed that Myc is sufficient to induce transformation of ovarian cells that are deficient for both Brca1 and p53 but not sufficient for the transformation of cells that are deficient for either Brca1 or p53. The transformed Brca1-deficient OSE cells display an increased number of centrosomes, acquire complex chromosome aberrations, and lack Rad51 nuclear foci in the presence of DNA-damaging agents, such as mitomycin C and cisplatin. Immunocompetent mice injected with transformed OSE cells develop tumors that resemble human metastatic serous ovarian carcinoma, the most common type of ovarian cancer in women. Consistent with the reported platinum chemosensitivity in patients with Brca1-associated ovarian cancer, the Brca1-deficient OSE cells have increased sensitivity to the DNA-damaging agent cisplatin, whereas sensitivity to the microtubule poison paclitaxel is similar between Brca1 wild-type and Brca1-deficient cells. The Brca1 wild-type and Brca1-deficient mouse ovarian tumors and cell lines provide a new experimental system for the evaluation of therapies that target the Brca1 pathway. (Cancer Res 2006; 66(18): 8949-53)

Introduction

The majority of hereditary ovarian cancers are of a serous type and can be attributed to germ-line mutations in the *Brca1* tumor suppressor gene. Hereditary and sporadic ovarian cancers are similar in many respects; however, patients with hereditary mutations in *Brca1* develop ovarian cancers earlier than patients with sporadic cancers (1), suggesting that the loss of the Brca1 function lowers the threshold for oncogenic transformation. The loss of heterozygosity for *Brca1* and the inactivation of the p53 function seem to be the early events in the induction of hereditary

ovarian cancer (2). Consistent with this, familial ovarian cancers have a significantly higher frequency of p53 mutations than sporadic cancers (3), indicating that the loss of p53 function is required for a cell to tolerate the loss of the Brca1 function (4). However, little is known about the requirement for additional genetic alterations that collaborate with *Brca1* and p53 in the transformation of ovarian surface epithelial (OSE) cells. Considering the distinct clinical, histopathologic, and molecular aspects of Brca1-associated ovarian cancers, there is a great need to generate ovarian cancer mouse models for the comparison of Brca1 wild-type and Brca1-deficient ovarian tumors.

Materials and Methods

Mouse strains. K5-TVA mice (5) were crossed with Brca1^{lox/lox} (6), p53^{lox/lox} (7), or p53^{-/-} (8) mice. Triple transgenic K5-TVA; Brca1^{lox/lox}; p53^{lox/lox} mice were generated by crossing K5-TVA; Brca1^{lox/lox} and K5-TVA; p53^{lox/lox} mice.

Retroviral constructs and generation of genetically defined ovarian cancer cell lines. Replication-competent avian leukosis virus long terminal repeat with splice acceptor (RCAS) retroviral constructs and viral infection of mouse ovarian explants have been described (5, 9). For the generation of C1, C11, C2, C22, and C3 cell lines, ovarian explants from K5-TVA; p53^{-/-} mice (5) were infected with different combinations of RCAS viruses carrying human *Myc*, mouse *K-ras*^{G12D}, and mouse myristoylated *Akt1* oncogenes: C1 and C11 (*Myc* plus *K-ras*), C2 and C22 (*Akt* plus *Myc*), and C3 (*Akt* plus *K-ras*). The BR2, BR5, and BR6 cell lines were derived by RCAS-*Cre* and RCAS-*Myc* infection of ovarian explants from mice with K5-TVA; Brca1^{lox/lox}; p53^{lox/lox} genetic background. Tumors from nude mice injected with the Brca1 wild-type cell lines C1, C11, C2, C22, and C3 were used to generate T1, T11, T2, T22, and T3 tumor cell lines, whereas tumors from nude mice injected with the Brca1-deficient cell lines BR2, BR5, and BR6 were used to generate the TBR2, TBR5, and TBR6 tumor cell lines.

Confirmation of gene recombination and expression. Genomic DNA extracted from different cell lines was used to detect the Cre-mediated recombination of the p53 and Brca1 genes. The primers used for the PCR and reverse transcription-PCR (RT-PCR) analyses are described in Supplementary Materials and Methods.

Tumor production and immunodetection of proteins. Tumor production, Western blotting, immunohistochemistry, and immunofluorescence were done as described previously (9). The antibodies used for immunodetection of proteins are described in Supplementary Materials and Methods.

Cell treatment with mitomycin C, cisplatin, and paclitaxel. Equal numbers of cells were plated into six-well dishes in DMEM/F12 with 10% FCS. After 1 day of growth in culture, the medium was replaced with a medium that contained 1 μg/mL mitomycin C (MMC; Roche, Indianapolis, IN) or gradually increasing concentrations of cisplatin (*cis*-diamminedichloroplatinum, Calbiochem, Darmstadt, Germany) or paclitaxel (Baccatin III *N*-benzyl-β-phenylisoserine ester, LC Laboratories, Woburn, MA). The cells were treated with MMC overnight or with cisplatin or paclitaxel for 8 days. Cell numbers were determined using a counting chamber (Hausser Scientific, Horsham, PA). To determine chromosome breakage, subconfluent cells were exposed to 1 μmol/L cisplatin for 48 hours and metaphase chromosomes were prepared as described previously by Litman et al. (10).

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Table 1. Identification of oncogenes that cooperate with the loss of p53 and/or Brca1 in inducing transformation of mouse OSE cells

Transgenic background	RCAS virus combination	Transformation
K5-TVA; Brca1 ^{lox/lox} ; p53 ^{lox/lox}	Cre AP	-
	Cre GFP	-
	Cre K-ras	-
	Cre HER-2	-
	Cre Akt	-
	Cre Myc	+
K5-TVA; p53 ^{lox/lox}	Cre Myc	-
K5-TVA; Brca1 ^{lox/lox}	Cre Myc	-

Results and Discussion

Myc cooperates with the loss of Brca1 and p53 in transforming mouse OSE cells. We have developed a K5-TVA-RCAS system in which defined multiple genetic alterations can be introduced into mouse OSE cells (5, 9), a single layer of cells that is believed to be the precursor for ovarian carcinoma. We have previously shown that the minimal requirement for the transformation of OSE cells from p53-deficient mice is a combination of any two of the following oncogenes: *Myc*, *K-ras*, and *Akt* (5). To identify the minimal requirement for the transformation of mouse OSE cells that are deficient for both Brca1 and p53, we crossed K5-TVA (5), Brca1^{lox/lox} (6), and p53^{lox/lox} (7) transgenic mice. The triple transgenic system allows for conditional inactivation of Brca1 and p53 in OSE cells as well as the introduction of putative oncogenes that are thought to collaborate with Brca1 and p53 in

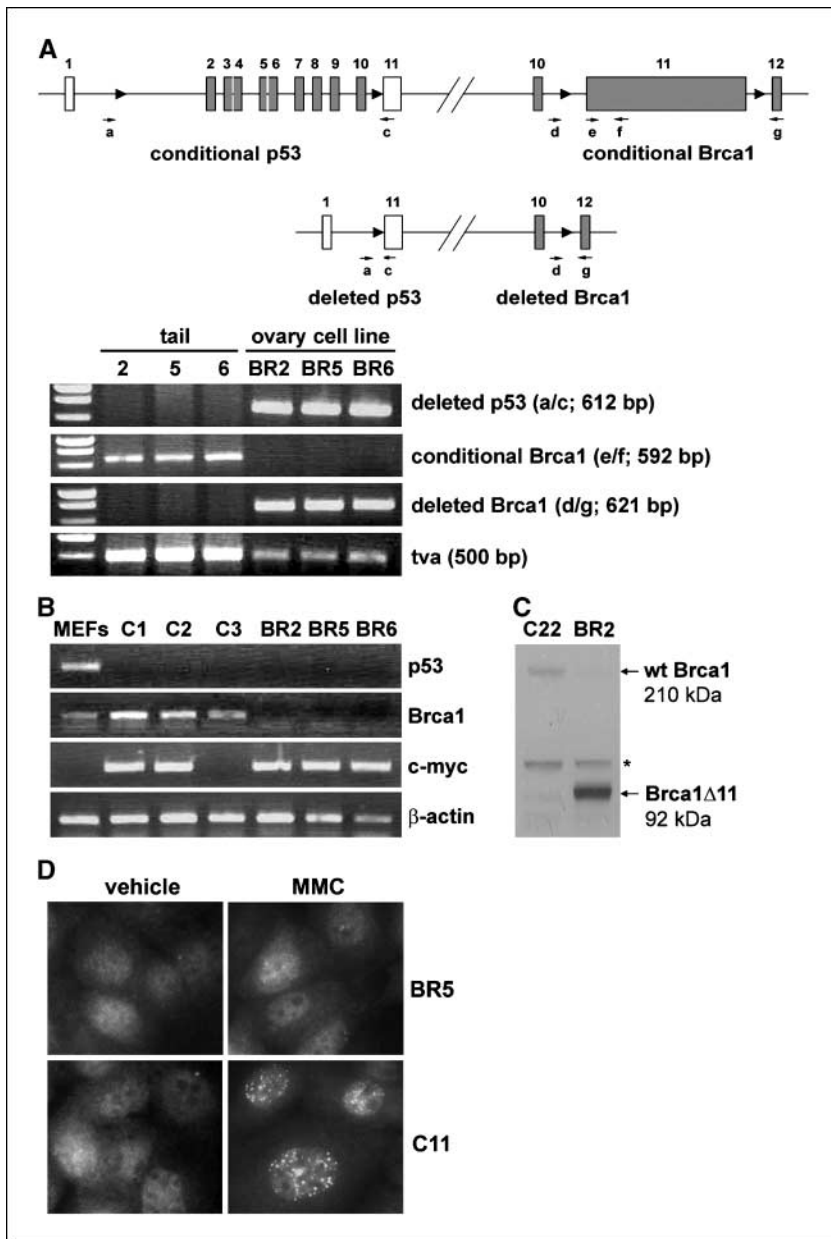


Figure 1. Characterization of genetically defined mouse ovarian cancer cell lines. *A*, detection of deleted *p53* (exons 2-10), conditional *Brca1*, deleted *Brca1* (exon 11), and *tva* alleles by PCR using DNA from mouse tails (2, 5, 6) and the corresponding transformed ovarian cell lines with deleted *p53* and *Brca1* tumor suppressor genes (BR2, BR5, and BR6). *B*, RT-PCR detection of *p53*, *Brca1*, *Myc*, and β -actin in MEFs and engineered mouse ovarian cancer cell lines C1 (*p53*^{-/-}; *Myc*; *K-ras*), C2 (*p53*^{-/-}; *Myc*; *Akt*), C3 (*p53*^{-/-}; *K-ras*; *Akt*), and BR2, BR5, and BR6 (*p53*^{-/-}; *Brca1*^{-/-}; *Myc*). *C*, Western blot of whole-cell extracts from *Brca1* wild-type (C22) and *Brca1*-deficient (BR2) cell lines with a mouse *Brca1* antibody. Asterisk, nonspecific band. *D*, immunodetection of Rad51 nuclear foci formation after overnight exposure of *Brca1* wild-type (C11) and *Brca1*-deficient (BR5) OSE cell lines to 1 μ g/mL MMC or vehicle.

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tumorigenesis. Ovarian explants isolated from K5-TVA; *Brca1*^{lox/lox}; *p53*^{lox/lox} transgenic mice were infected *in vitro* with RCAS-*Cre* along with RCAS vectors carrying *K-ras*, *HER-2*, *Akt*, or *Myc* genes. We determined that the *Myc* oncogene is capable of transforming cells that are deficient for both *Brca1* and *p53*, whereas *K-ras*, *HER-2*, and *Akt* are not sufficient for the transformation of these cells (Table 1). *Myc*, however, is not sufficient for the transformation of ovarian cells with intact *Brca1* or *p53* (Table 1).

The mechanism by which *Myc* transforms *Brca1*- and *p53*-deficient mouse OSE cells is presently unknown. Tumor growth requires the activation of proliferative signals and amplification of the *Myc* gene might be sufficient to provide these signals. However, other oncogenes, such as activated *Akt*, *K-ras*, and *HER-2*, which are known to induce strong proliferative signals, were not sufficient for the transformation of the *Brca1*- and *p53*-deficient OSE cells in our assay. This suggests that *Myc* may play a different and possibly more direct role in the transformation of mouse OSE cells that are deficient for *Brca1* and *p53*. *Brca1* physically associates with *Myc* and represses its transcriptional and transformative activity (11). It has also been shown in transgenic mice that deregulated *Myc* expression leads to DNA damage and genomic instability, which induces *p53* activation and apoptosis (12). However, in the absence of the *p53* function, rather than leading to apoptosis, *Myc*-induced DNA damage may lead to uncontrolled genomic instability and cell proliferation. The amplification of *Myc* and the loss of the *p53* function often accompany the *Brca1* mutation in human and mouse breast tumors (13, 14), providing additional support for the role of *Myc* and *p53* in *Brca1*-associated tumor progression. Use of high-throughput technology, such as microarray analysis and comparison of OSE cell lines with different genetic alterations, might shed light on the cooperative pathways among *Myc*, *p53*, and *Brca1*.

Serous ovarian carcinomas develop in mice injected with *Brca1* wild-type and *Brca1*-deficient mouse OSE cells. Three independent OSE cell lines, designated as BR2, BR5, and BR6, were generated by infection of the ovaries from K5-TVA; *Brca1*^{lox/lox}; *p53*^{lox/lox} mice with a combination of RCAS-*Cre* and RCAS-*Myc*. PCR of mouse tail DNA and the corresponding transformed OSE cell line DNA was used to confirm the deletion of exons 2 to 10 in *p53* and exon 11 in *Brca1* in the transformed OSE cell lines (Fig. 1A). Because both *p53* and *Brca1* are located on chromosome 11, Cre-loxP-mediated recombination may result in recombination between *p53* and *Brca1* loci, potentially influencing carcinogenesis. PCR with four combinations of primers (Fig. 1) flanking *p53* (Fig. 1A, a and c) and *Brca1* (Fig. 1A, d and g) loxP sites did not detect such alterations (data not shown). OSE cell lines that are wild-type for *Brca1* were generated by the infection of ovarian explants from K5-TVA; *p53*^{-/-} mice with combinations of RCAS vectors carrying *Myc*, *K-ras*, and *Akt* oncogenes as described previously (5, 9). RT-PCR (Fig. 1B) was used to detect the presence of *p53*, *Brca1*, *Myc*, and β -actin in mouse embryonic fibroblasts (MEFs), mouse ovarian cancer cell lines deficient for *p53* and wild-type for *Brca1* (C1, C2, and C3), and mouse ovarian cancer cell lines deficient for both *Brca1* and *p53* (BR2, BR5, and BR6). A Western blot of whole-cell extracts from *Brca1* wild-type and *Brca1*-deficient (exon 11 deleted) cell lines with a *Brca1* antibody shows expression of the 210-kDa wild-type *Brca1* and the 92-kDa *Brca1* ^{Δ 11} spliced product (Fig. 1C). *Brca1* is known to play a role in the fidelity of centrosome duplication (15) and localization of DNA repair proteins, such as Rad51, to the DNA damage-induced foci (16). Consistent with the requirement for *Brca1* in these processes, *Brca1*-deficient cell lines displayed an increased number of

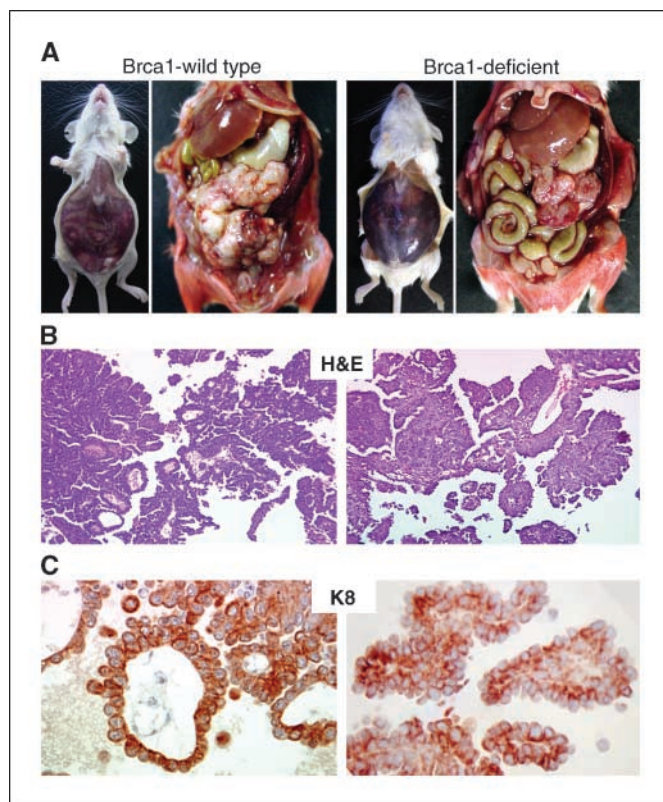


Figure 2. Phenotypic properties of transformed ovarian cells and tumors that are wild-type or deficient for *Brca1*. *A*, ascites accumulation and carcinomatosis are apparent 4 to 10 weeks after i.p. injection of the *Brca1* wild-type C2 cells ($n = 10$) and 10 to 14 weeks after i.p. injection of the *Brca1*-deficient BR5 cells ($n = 16$) into FVB mice. *B*, *Brca1* wild-type and *Brca1*-deficient mouse tumors resemble human ovarian serous papillary carcinoma. H&E staining. *C*, *Brca1* wild-type and *Brca1*-deficient mouse tumors are positive for the epithelial marker keratin 8 (*K8*).

centrosomes (Supplementary Fig. S1) and lacked Rad51 nuclear foci in the presence of the DNA cross-linking agent MMC (Fig. 1D).

I.p. injection of *Brca1* wild-type and *Brca1*-deficient cell lines into nude or FVB mice invariably resulted in metastatic tumor formation. Similar to human ovarian tumors, the mouse tumors were associated with the accumulation of hemorrhagic ascites and bloating (Fig. 2A). The tumor nodules grew on the surfaces of organs without significant invasion. Tumor spread sites included the mesothelial lining of the peritoneum, intestines, pancreas, and diaphragm, whereas the liver, spleen, and kidneys were typically spared from tumor spread. Both *Brca1* wild-type and *Brca1*-deficient tumors were characterized by papillary structures typical of serous papillary carcinoma (Fig. 2B), which is the most common type of hereditary ovarian cancer. The OSE cell origin of the tumors was confirmed by the presence of the epithelial cell marker keratin 8 (Fig. 2C). The cell lines that were derived from the transformed primary ovarian epithelial cells and the mouse tumors maintained the typical epithelial cobblestone morphology of OSE cells (Supplementary Fig. S2).

Loss of *Brca1* function increases sensitivity of mouse OSE cells to the DNA-damaging agent cisplatin. Administration of the microtubule poison paclitaxel and the DNA-damaging agent cisplatin is a current standard of postoperative chemotherapy for patients with advanced ovarian cancer. Patients with hereditary ovarian cancers display a longer recurrence-free interval following

chemotherapy and have a longer overall survival rate (1). This phenomenon is likely due to induced chromosomal instability, cell cycle arrest, and subsequent apoptosis in cells that lack Brca1 and thus cannot repair the induced DNA damage. To test whether Brca1 status influences the sensitivity of OSE cells to drugs with different mechanisms of action, we compared the sensitivity of Brca1 wild-type and Brca1-deficient OSE cells to paclitaxel and cisplatin. OSE cell lines that are wild-type (C1, C2, C3, T1, T2, and T3) or deficient for Brca1 (BR2, BR5, BR6, TBR2, TBR5, and TBR6) were tested for their rate of survival in increasing concentrations of paclitaxel and cisplatin at doses that are effective in human ovarian cancer cell lines that harbor defined genetic alterations in genes such as *Brca2*, *p53*, *K-ras*, *TGFβ-RII*, and *CDNK2A* (17). We showed that transformed Brca1 wild-type and Brca1-deficient mouse OSE cell lines have a similar sensitivity to the microtubule poison paclitaxel (Fig. 3A). However, the DNA-damaging agent cisplatin was significantly more effective in

inducing cell death in the Brca1-deficient cell lines (Fig. 3A), presumably because these cells are unable to repair cisplatin-induced DNA damage. The exposure of ovarian cancer cell lines lacking functional Brca1 to 1 μmol/L cisplatin for 48 hours resulted in chromatid breaks and other complex chromatin aberrations, such as triradial and quadriradial chromosomes (Fig. 3B). We hypothesized that, in this case, Brca1 functioned as a chemotherapy sensitivity modulator independent of oncogenic composition. Consistent with this hypothesis, we showed that the introduction of *Akt* or *H-ras* into the Brca1-deficient TBR5 cell line results in a similar response to cisplatin or paclitaxel compared with that of TBR5 cells with an empty vector (Supplementary Fig. S3). Similarly, it has been shown that the *K-ras* status in human ovarian cancer cell lines does not affect chemosensitivity to paclitaxel and cisplatin (17, 18).

Although these studies were done in culture, our results concur with previous findings that Brca1-deficient human ovarian cancer cells have a greater sensitivity to cisplatin *in vitro* (16) and that

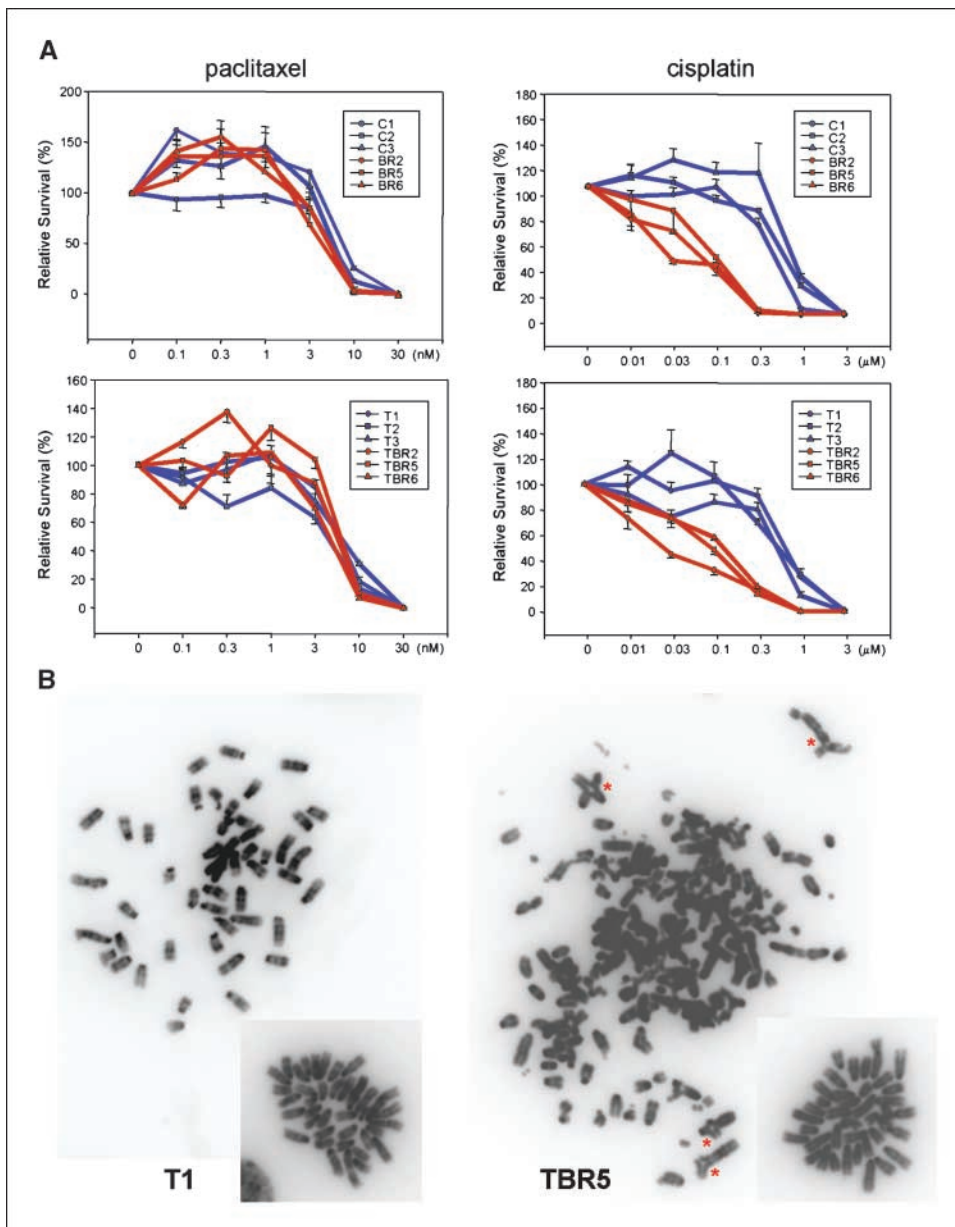


Figure 3. Sensitivity of Brca1 wild-type and Brca1-deficient cells to drugs with differential mechanisms of action. A, sensitivity of Brca1 wild-type (C1, C2, C3, T1, T2, and T3) and Brca1-deficient (BR2, BR5, BR6, TBR2, TBR5, and TBR6) cell lines to the microtubule poison paclitaxel and the DNA-damaging agent cisplatin. Results are ratio of the number of drug-treated cells to that of control cells. Points, mean of triplicate experiments; bars, SD. B, metaphase chromosome analysis of Brca1 wild-type (T1) and Brca1-deficient (TBR5) cell lines left untreated (*inset*) or exposed to 1 μmol/L cisplatin for 48 hours (*main*). Asterisks, triradial or quadriradial chromosomes.

patients with Brca1-associated ovarian carcinoma respond better to platinum-based therapies (1, 19). The increased sensitivity of the Brca1-deficient OSE cells to cisplatin and the reported platinum chemosensitivity displayed by patients with Brca1-associated ovarian cancer raise the question about whether platinum agents would be effective in the treatment of patients with Brca1-associated breast cancer. This biochemical difference between Brca1 wild-type and Brca1-deficient cells provides an opportunity to develop novel therapeutic agents that generate specific DNA lesions that require functional Brca1 for their repair. The compelling factor of this type of therapy is its selectivity; the nontumor cells that are heterozygous for *Brca1* should have normal Brca1 function and, therefore, normal levels of DNA repair, whereas the Brca1-deficient tumor cells should be highly sensitive to DNA-damaging treatments (20). Unlike cell lines isolated from human tumors, the Brca1 wild-type and Brca1-deficient mouse OSE cell lines are capable of forming serous papillary carcinomas in immunocompetent mice, thus providing a unique experimental

model to study the sensitivity of ovarian tumors to various therapies that may interfere with the immune system. We anticipate that this mouse model will facilitate the evaluation of various therapies that target the Brca1 pathway as well as assist in the identification of novel biochemical pathways that are associated with hereditary ovarian cancers.

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